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Binding of *p*-Nitrophenyl α -D-Galactopyranoside to *lac* Permease of *Escherichia coli*

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ABSTRACT: Binding of the substrate analogue *p*-nitrophenyl α -D-galactopyranoside (NPG) to *lac* permease of *Escherichia coli* in different membrane preparations was investigated. Binding was assayed with an improved version of the centrifugation technique introduced by Kennedy et al. [Kennedy, E. P., Rumley, M. V., Armstrong, J. B. (1974) *J. Biol. Chem.* 249, 33-37]. Two binding sites for NPG were found with dissociation constants of about 16 μ M and 1.6 mM at pH 7.5 and room temperature. With purified *lac* permease reconstituted into proteoliposomes, it could be shown that one permease molecule binds two substrate molecules. Oxidation of *lac* permease with the lipophilic quinone plumbagin or alkylation with the sulfhydryl reagent *N*-ethylmaleimide caused a 12-fold increase in the first dissociation constant. The second dissociation constant seemed to be increased as well, but its value could not reliably be estimated. Ethoxyformylation of *lac* permease with diethyl pyrocarbonate totally abolished NPG binding. The implications of these results for the catalytic performance of the enzyme are discussed.

lac permease is responsible for the transport of lactose across the cytoplasmic membrane of *Escherichia coli*. By coupling the translocation of lactose and proton, accumulation of lactose

in the cell against a concentration difference is achieved at the expense of free energy stored in the electrochemical potential difference for protons. Mechanistic studies on the carrier have been carried out on *E. coli* membrane vesicles containing *lac* permease and other components in the active state and on the enzyme purified to homogeneity and reconstituted into proteoliposomes [for a review, see Kaback (1986)]. These systems enabled detailed investigations on the kinetic

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performance of the enzyme (Robertson et al., 1980; Garcia et al., 1983; Viitanen et al., 1983). Further insight into the catalytic process was sought by modifying the enzyme at the single amino acid level by means of site-directed mutagenesis (Kaback, 1988). Despite of all these efforts, a detailed picture of the catalytic cycle which can explain all experimental findings still eludes us.

In terms of enzyme catalysis, *lac* permease transforms two substrates, external lactose and protons, into two products, internal lactose and protons, without chemical modification. Substrates must bind to a particular conformation of the enzyme with binding sites accessible only from the outside. Similarly, the release of the products has to occur from conformations which open the binding sites to the inside only; this implies at least one transition step between the two types of conformations. Since no chemical reaction is involved, measurements of binding of substrates and products to the enzyme under equilibrium conditions can be easily performed. The overall dissociation constants obtained comprise the intrinsic dissociation constants for the ligands and the equilibrium constants for conformational transitions. Therefore, in this case, results of binding assays do not just give information on the binding affinities but also on the translocation equilibria.

Binding of ligands other than protons to *lac* permease was first measured indirectly by Carter et al. (1968). Dissociation constants were deduced from the concentration dependence of the protection of ligands against inactivation of the enzyme by *N*-ethylmaleimide (NEM).¹ Later Kennedy et al. (1974) measured binding directly by determining the amount of radioactively labeled ligand in the pellet of membrane vesicles after equilibration and centrifugation. The interstitial volume in the pellet together with the internal volume of the vesicles was assayed with [³²P]phosphate. This binding assay, however, was not pursued but replaced by a method where the depletion of free ligand due to binding is measured by means of flow dialysis (Rudnick et al., 1976; Teather et al., 1980). We have returned to the centrifugation technique because of its higher sensitivity and have improved considerably its performance by changing the protocol. This was necessary since data recently obtained (Lolkema et al., 1990) suggest that *lac* permease has more than one binding site for lactose. Moreover, mutations of the enzyme frequently cause an increase in dissociation constants to such an extent that binding can hardly be measured with flow dialysis.

Any binding assay is restricted to the concentration range of ligand where bound ligand is a measurable fraction of total ligand. The maximal concentration of bound ligand is determined by the concentration of permease to be achieved in that step of the assay where binding is actually measured. Hence, binding of only those ligands can be assessed whose dissociation constants are of the order of this permease concentration. This unfortunately excludes lactose whose dissociation constant is most likely of the order of millimolar. However, *p*-nitrophenyl α -D-galactopyranoside (NPG), whose dissociation constant was reported to range between 10 and 20 μ M, is a suitable ligand. The higher sensitivity of the binding assay will be demonstrated through the effect of chemical modifications of the enzyme on NPG binding. Moreover, it will be shown that *lac* permease indeed has two binding sites for NPG. The implication of the results for the

catalytic cycle will be discussed.

MATERIALS AND METHODS

Materials. [1-¹⁴C]Lactose was purchased from Amer-sham/Searle. [¹⁴C]Urea and [³H]water were obtained from New England Nuclear. *p*-Nitro[6-³H]phenyl α -D-galactopyranoside was synthesized by Yu-Ying Liu of the Isotope Synthesis Group, Hoffmann-La Roche Inc., under the direction of Arnold Liebman. Valinomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were obtained from Calbiochem. Nigericin was generously provided by Dr. John Wesley, Hoffmann-La Roche Inc. All other materials were reagent grade, obtained from commercial sources.

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* strain T206, which carries the *lacY* gene in the recombinant plasmid pACYC184, was grown and induced as described by Teather et al. (1980). Right-side-out (RSO) membrane vesicles were prepared as described (Kaback, 1971) and stored in 100 mM potassium phosphate, pH 7.5, and 1 mM MgSO₄ in liquid nitrogen. Purified RSO membrane vesicles were obtained by layering 6 mL of vesicle suspension of 1 mg of protein/mL in 100 mM potassium phosphate, pH 7.5, 10 mM EDTA, and 5 mM dithiothreitol (DTT) on top of 20 mL of a 50% (w/v) sucrose solution in the same buffer. After centrifugation for 45 min at 50000g in a Sorval SS-34 rotor, the vesicles at the interface were collected, diluted 100 times in the same buffer, and centrifuged for 45 min at 8000g in a Sorval GSA rotor. The vesicles were then washed with 100 mM potassium phosphate, pH 7.5, and 1 mM MgSO₄ and stored in the same buffer in liquid nitrogen. Inside-out (ISO) membrane vesicles were prepared as described (Reenstra et al., 1980) and stored in 50 mM potassium phosphate, pH 7.5, in liquid nitrogen. Membrane protein was estimated according to Lowry et al. (1951) using bovine serum albumin as a standard.

Purification and Reconstitution of *lac* Permease. *lac* permease was purified and reconstituted as described by Viitanen et al. (1986) except for the following modifications. The permease-containing fractions from the DEAE-Sepharose column were diluted immediately without addition of extra lipids. This resulted in a protein to lipid ratio of about 400 as compared to about 30000 usually chosen for reconstitution experiments (Garcia et al., 1983). The proteoliposomes were not sonicated and were stored in 50 mM potassium phosphate, pH 7.5, in liquid nitrogen. The concentration of permease was determined by quantitative amino acid analysis.

Binding Assay. Crude RSO or purified RSO membrane vesicles were diluted to a concentration between 1.5 and 6 mg of protein/mL in 100 mM potassium phosphate, pH 7.5, and 1 mM MgSO₄. Unless otherwise stated, CCCP (final concentration 5 μ M), [³H]NPG (4 Ci/mmol, final concentration between 1.5 and 3 μ M), and [¹⁴C]urea (1.2 mCi/mL) were added to the suspension. It was then split into two equal parts. One part was made 200–300 μ M in HgCl₂ with a 25 mM HgCl₂ solution. An equal volume of water was added to the other part. The latter is used to measure the total binding and the former to measure the unspecific binding (see Results). Aliquots of 95 μ L of the suspensions were then placed into 1.5-mL Eppendorf centrifugation tubes, and 5 μ L of appropriate stock solutions of unlabeled NPG was added in order to obtain the desired total NPG concentrations. The content of each tube was thoroughly mixed by vortexing and incubated for at least 1 h at room temperature. A 10- μ L aliquot was then taken from each tube and diluted with 1 mL of water in a scintillation vial by repeatedly rinsing the pipet tip in the water. The tubes were centrifuged for 15 min at

¹ Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; pCMBS, *p*-(chloromercuri)benzenesulfonate; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; NPG, *p*-nitrophenyl α -D-galactopyranoside; plumbagin, 5-hydroxy-2-methyl-1,4-naphthoquinone; TDG, β -D-galactopyranosyl-1-thio- β -D-galactopyranoside; RSO, right-side-out; ISO, inside-out.

10000g in an Eppendorf 5412 tabletop centrifuge. A 10- μ L aliquot was carefully taken from the supernatant and transferred to a scintillation vial as described before. The remainder of the supernatant was withdrawn in several steps with a pipet, great care being taken that nothing of the pellet was removed. The pellet was resuspended in 90 μ L of fresh buffer by vortexing, and a 10- μ L aliquot of the resuspension was transferred to a scintillation vial as described. Each vial received 10 mL of scintillation cocktail and was counted 3 times in two channels set for [3 H]/[14 C] double labeling.

The same procedure was used for ISO membrane vesicles and proteoliposomes except for the following modifications. The vesicles or liposomes were suspended in 50 mM potassium phosphate, pH 7.5, which contained 12.5 μ M valinomycin and 1.25 μ M nigericin instead of CCCP. The 95- μ L aliquots were placed into airfuge tubes and, after addition of NPG and equilibration, were centrifuged for 45 min in a Beckman airfuge operated at a pressure of 27 psi (160000g). After removal of the supernatant, the pellet was dissolved in 100 μ L of a 5% SDS solution by intermittent vortexing. The solution was then transferred to an empty scintillation vial, and the tube was rinsed in several steps with 1 mL of water which was also added to the vial. The vials that contained the aliquots from the total sample and the supernatant received 100 μ L of 5% SDS prior to the addition of scintillation cocktail.

Addition of 200 μ M HgCl_2 to the proteoliposomes to measure the nonspecific binding caused them to aggregate and eventually precipitate. Decreasing the concentration to 20 μ M (which is about 10 times the permease concentration) prevented this phenomenon, but the effect of HgCl_2 on NPG binding became time dependent. An immediate drop by 80% of the initial binding after addition of HgCl_2 was followed by a slow decrease which leveled off after 6 h. In contrast, NPG binding to proteoliposomes in the absence of HgCl_2 was found to be constant for over 8 h, thus eliminating the possibility that proteoliposomes would slowly degrade over prolonged periods of time. The slow phase then most likely reflects the permeation of Hg^{2+} ions through the multilayered liposomes. The proteoliposomes were not sonicated after reconstitution to assure a quantitative collection of the permease in the pellet. In order to ascertain that the final value correctly reports unspecific binding of NPG to proteoliposomes, we made use of the fact that extensive DEPC treatment totally abolishes specific binding. Titration of NPG binding up to 2 mM NPG with proteoliposomes which were incubated with 20 μ M HgCl_2 for 6 h yielded essentially the same results as titrations with DEPC-treated proteoliposomes incubated for 6 h either with DTT or with 20 μ M HgCl_2 .

The proteoliposomes contained a small amount of ^3H due to the photolabeling of the permease with [^3H]NPG in the purification procedure. It was determined by performing the binding assay without addition of [^3H]NPG and [^{14}C]urea. The cpm values found in the pellet were subtracted from those obtained in the binding experiments (this correction never exceeded 10%).

Evaluation of Data. Judged by the external standard technique, quenching was fairly constant for all samples. A quench curve could therefore be replaced by two standards of identical composition but with known amounts of [^3H]NPG or [^{14}C]urea. The cpm values measured in the two channels were then converted into dpm values for [^3H]NPG and [^{14}C]urea by using the efficiencies determined with the two standards.

The pellet volume and the concentration of bound NPG were calculated from the ^3H and ^{14}C dpm values in the samples

taken from total, supernatant, and pellet by means of eq A7–A9 given in the Appendix. Values for bound NPG, c_b , can be calculated by using data from total sample and pellet, from supernatant and pellet (eq A8 with $x = t$ and $x = s$, respectively) and from total sample and supernatant (eq A9). Usually, the first two agreed within experimental error, and the average value of the two is plotted in the figures. In contrast, the same values for c_b calculated with eq A9 agreed only with those from eq A8 when the total concentration was small (in the order of a few micromolar).

The evaluation included a calculation of the mass balance for NPG and urea (equations not shown) in order to detect improper performance of the experimental procedure (e.g., a loss in pellet when removing the supernatant). The standard deviation $s(y)$ of any quantity $y = f(x_1, x_2, \dots)$ computed from the uncorrelated quantities x_1, x_2, \dots was estimated as $s(y) = \{\sum s(x_i)^2 (\partial f / \partial x_i)^2\}^{1/2}$ where $s(x_i)$ is the standard error of x_i (when available) or its estimated experimental error (McIntosh & McIntosh, 1980).

Estimation of values for binding parameters was performed with the nonlinear fitting program MODFIT as described by McIntosh and McIntosh (1980). The functions describing the binding curves, i.e., bound NPG as a function of the total NPG concentration, are given in the Appendix (eq A11–A14). In cases where saturation of the unspecific binding had to be taken into account (eq A10 instead of A11), the following iterative procedure was applied to calculate the concentration c_f of free NPG. The value for the total NPG concentration was first assigned to c_f in the denominator of eq A10, and c_f was calculated with eq A14. The value thus obtained was then assigned to c_f in the denominator of eq A10; a new value for c_f was calculated and so on until the relative change in values for c_f from successive iterations was less than 10^{-12} .

Weight $1/s(c_b)^2$ was used in MODFIT which takes into account that the accuracy of c_b decreases with increasing c_t . In case of a Scatchard analysis (see eq A15 in the Appendix), an intermingling of independent (c_f) and dependent variable (c_b) occurs in the calculations of free and bound NPG which introduces a complex weighing of the data. Therefore, weight 1 was used in MODFIT.

RESULTS

Binding Assay. The protocol of the binding assay is outlined in Figure 1. Membrane vesicles containing *lac* permease are equilibrated with radiolabeled ligand. The total ligand concentration is made up of the concentrations of bound and free ligand. Spinning down the vesicles quantitatively does not disturb the equilibrium condition and leaves only the free ligand in the supernatant. Measurement of the radioactivity in samples taken before and after centrifugation allows for an estimation of the free and bound concentrations in the original sample only if the bound concentration is a significant fraction of the total concentration. A much higher sensitivity is achieved when, instead of the supernatant, the pellet is used in the analysis. The pellet contains all the bound ligand and only a small fraction of the initially present free ligand. The latter is determined by the volume of the pellet. The volume of the pellet is estimated by means of a marker substance carrying a second radioactive label which partitions evenly into all water phases and does not bind to the membranes. A quantitative evaluation of the assay is given in the Appendix.

Binding vs Accumulation. The binding assay described above assumes that the ligand concentration inside the vesicles is equal to the concentration in the suspending medium. Therefore, no electrochemical potential difference for protons across the membrane should exist, since *lac* permease catalyzes

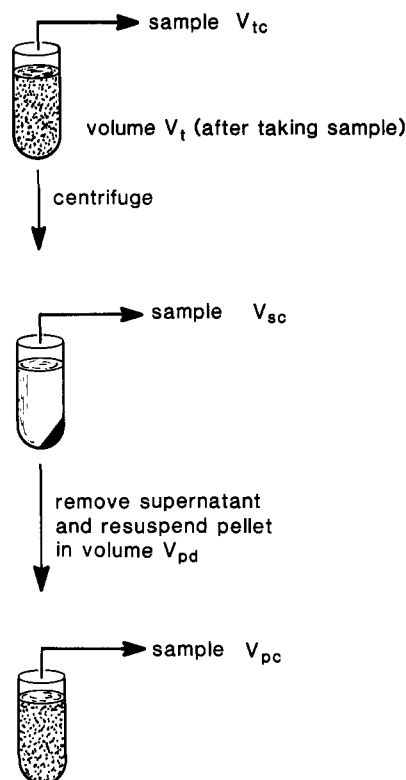


FIGURE 1: Protocol of the binding assay. Samples are taken from the initial membrane suspension (V_{tc}), from the supernatant (V_{sc}), and from the pellet resuspension (V_{pc}). Radioactivity is then assayed as described under Materials and Methods. For further explanations, see the text.

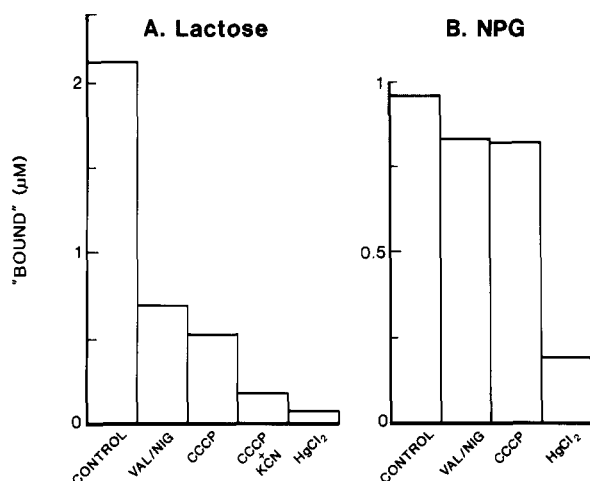


FIGURE 2: Accumulation and binding of lactose (A) and NPG (B) in RSO membrane vesicles at pH 7.5. In the control samples, CCCP was omitted while additions were 19 μ M valinomycin plus 1.9 μ M nigericin, 7.5 μ M CCCP, 7.5 μ M CCCP plus 10 mM KCN, and 300 μ M HgCl₂ as indicated. Concentration of membrane protein was 4.7 mg/mL (A) and 5.6 mg/mL (B). Lactose binding was measured with 34 μ M [¹⁴C]lactose (59.6 mCi/mmol) and [³H]water (4.5 mCi/mL) as volume marker; total NPG concentration was 7 μ M.

symport of galactosides with protons. Although the membrane is quite permeable to NPG, it is not guaranteed that passive diffusion of NPG will eventually collapse an existing potential difference. Therefore, we have used lactose, to which the membranes are much less permeable, in order to test for the existence of a potential difference. Crude RSO membrane vesicles displayed a considerable amount of binding which, however, could be greatly decreased by adding uncouplers such as valinomycin plus nigericin or CCCP (Figure 2A). Surprisingly, addition of an electron transport inhibitor (CN⁻) on top of CCCP reduced binding even more and almost to the

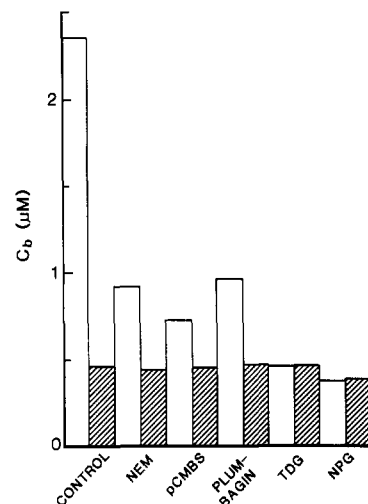


FIGURE 3: Binding of NPG to purified RSO membrane vesicles in the absence (open bars) and presence (hatched bars) of 200 μ M HgCl₂ at pH 7.5. Where indicated, the vesicles were treated for at least 1 h with 10 mM NEM, 0.5 mM pCMBS, and 100 μ M plumbagin, respectively, or the samples contained, in addition to labeled NPG, 10 mM TDG or 10 mM unlabeled NPG. Concentrations of total labeled NPG and membrane protein were 35 μ M and 2.2 mg/mL, respectively.

level found when membranes were treated with HgCl₂ (Figure 2A). It will be shown below that HgCl₂ totally annihilates binding of ligands to *lac* permease. Obviously, the "binding" of lactose found in the absence of HgCl₂ is mainly accumulation. As expected, accumulation was substantially lower for NPG (Figure 2B) but still large enough to interfere with binding. Purification of RSO membrane vesicles (see Materials and Methods) yielded a preparation which showed identical binding of lactose in the presence and absence of HgCl₂. This is consistent with the notion that the dissociation constant for lactose binding is in the millimolar range (Wright et al., 1981) for which the assay, with these membranes, is not sensitive enough. The purification removes residual cells and spheroplasts which apparently have enough endogenous energy sources to maintain an electrochemical potential difference for protons even in the presence of CCCP and CN⁻. Any residual electrochemical potential difference in purified RSO membrane vesicles was abolished by uncouplers as evidenced by a small drop in NPG binding upon addition of CCCP or valinomycin plus nigericin (data not shown). Neither ISO membrane vesicles nor proteoliposomes are contaminated with cells or spheroplasts because of different preparation procedures. The addition of uncouplers guaranteed equilibrium conditions.

Nonspecific Binding. In addition to specific binding to *lac* permease, NPG is likely to bind nonspecifically to proteins and lipids in the membrane due to the lipophilic nitrophenyl moiety. We have checked this possibility by measuring binding to membranes devoid of *lac* permease. *E. coli* strain T184 harboring the vector pACYC184 without the *lacY* insert was grown and induced as strain T206 (see Materials and Methods). RSO vesicles prepared from these cells indeed bind NPG. The binding was proportional to the total NPG concentration with $k_{ub} = 0.022$ (see eq A11 in the Appendix). In order to assess this nonspecific binding to membranes containing *lac* permease, the specific binding to the enzyme has to be eliminated. Modification of *lac* permease with the sulfhydryl reagents NEM and pCMBS as well as with the lipophilic oxidant plumbagin did reduce the binding of NPG; however, a further decrease in binding to a constant level was observed in all cases upon nonspecifically poisoning the enzyme

Table 1: Dissociation Constants (K_{L1} , K_{L2}) and Binding Capacity (C_{sb}) for Binding of NPG to *lac* Permease as Well as Average Dissociation Constant (K_{ub}) and Capacity ($C_{ub} = k_{ub}K_{ub}$) for Nonspecific Binding in Different Membrane Preparations at pH 7.5 and Room Temperature

prepn (no. of expt.) ^a	membrane preparation	K_{L1} (μ M)	K_{L2} (mM)	C_{sb} (μ M)	$k_{ub} \times 1000$	K_{ub} (mM)
1 (1)	purified RSO vesicles	16.0 (1.1) ^c	1.35 (0.25)	3.52 (0.28)	14.9 (0.2)	<i>b</i>
	+NEM	190 (10)	ND ^e	3.52 (0.28)	14.9 (0.2)	
2 (1)	purified RSO vesicles	16.0 (0.9)	2.61 (1.22)	3.38 (0.12)	14.2 (0.2)	7.55 (1.96)
	+plumbagin	193 (10)	~8.2	3.38 (0.12)	14.2 (0.2)	7.55 (1.96)
3 (1)	purified RSO vesicles	16.3 (0.6)	1.28 (0.39)	3.08 (0.09)	13.4 (0.1)	
4 (2)	ISO vesicles	16.1 (0.4)	2.90 (1.2)	0.99 (0.02)	6.34 (0.04)	
5 (2)	proteoliposomes	21.2 (0.6)	1.59 (0.28)	1.56 ^d (0.03)	2.96 (0.02)	14.2 (2.0)
6 (3)	proteoliposomes	19.2 (0.4)	1.69 (0.34)	1.75 (0.03)	5.00 (0.05)	6.54 (0.74)

^a Number of independent titrations whose results could be combined in one data set for estimating parameter values. ^b Nonspecific binding was proportional to the total NPG concentration (cf. eq A11). ^c Standard deviation. ^d Concentration of *lac* permease was $1.63 \pm 0.05 \mu\text{M}$ as determined by quantitative amino acid analysis. ^e Not determined.

with HgCl_2 (Figure 3). These changes were not caused by an effect of HgCl_2 on the other proteins and lipids in the membrane since binding of NPG to membranes devoid of *lac* permease was not affected by HgCl_2 .

Nonspecific binding has usually much lower affinities but higher capacities with respect to specific binding. Hence, it should be possible to expel the specifically bound NPG from *lac* permease by means of a high concentration of a second ligand. Addition of the ligand TDG (final concentration 10 mM, which is about 130 times more than its dissociation constant) indeed reduced NPG binding and exactly to the level observed in the presence of HgCl_2 (Figure 3). Since NPG binding was measured with the labeled ligand, unlabeled NPG can also be used as a competing ligand. Addition of 10 mM cold NPG again depressed binding to the level in the presence of HgCl_2 which, however, is somewhat lower than that for the other conditions in Figure 3, thus indicating that nonspecific binding is saturable, too.

In view of the evidence presented above, we concluded that treatment of membrane vesicles with HgCl_2 abolishes only specific binding of NPG to *lac* permease and therefore have used this procedure routinely to assess nonspecific binding.

Titration of NPG Binding to RSO and ISO Membranes. The dependence of the concentration of NPG bound to RSO membrane vesicles on the total NPG concentration is presented in Figure 4 (O). The nonspecific binding (X) was proportional to the total concentration. NPG bound specifically to *lac* permease was estimated from the difference between total and nonspecific binding and plotted according to Scatchard (1949) in Figure 5 (O). A systematic deviation from a straight line pertinent to a single binding site was found above a bound concentration of $3 \mu\text{M}$. It thus appears that the analysis of the data has to include a second binding site for NPG (see Appendix). Estimation of parameters as described under Materials and Methods yielded a reproducible value of $16 \mu\text{M}$ for the dissociation constant of the high-affinity binding site in different preparations. The values for the second site were less accurate and ranged between 1 and 3 mM (see Table I, preparations 1–3).

Similar results were obtained for the titration of NPG binding to ISO membrane vesicles (data not shown), and the corresponding values for the binding parameters of two independent titrations of the same preparation are given in Table I (preparation 4). Nonspecific binding to these membranes was about 2 times smaller than to RSO vesicles, but this was counterbalanced by a 3-fold decrease in permease concentration. Moreover, it turned out to be rather difficult to collect the much smaller ISO vesicles quantitatively in a pellet even with the airfuge which caused a larger scatter in the data. Thus, except for the proof that NPG binds similarly to *lac* permease, ISO membrane vesicles did not offer any advantage

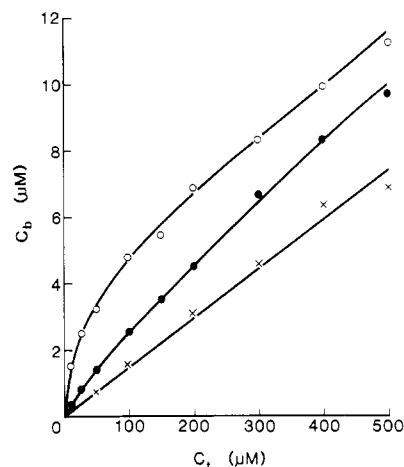


FIGURE 4: Titration of NPG binding to purified RSO membrane vesicles at pH 7.5. The concentration of bound NPG was determined as described in the text and is plotted vs the total NPG concentration for untreated vesicles (O), for vesicles incubated with 10 mM NEM (●), and for vesicles in presence of $200 \mu\text{M}$ HgCl_2 (X). The concentration of membrane protein was 2 mg/mL . The curves are calculated with eq A11–A14 (see the Appendix). The parameter values are listed in Table I (preparation 1).

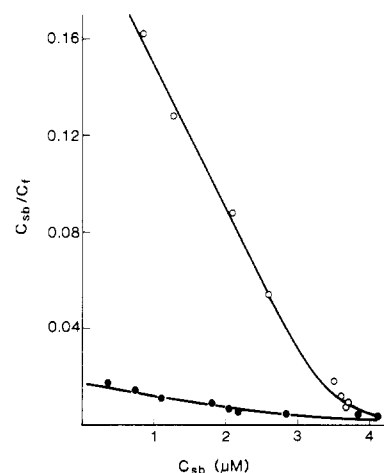


FIGURE 5: Scatchard plot of NPG binding to reduced and oxidized *lac* permease at pH 7.5. The values for the concentration of specifically bound NPG and free ligand were calculated from titrations of purified RSO membrane vesicles treated with 1.7 mM DTT (O) or $43 \mu\text{M}$ plumbagin (●), and in the presence of $250 \mu\text{M}$ HgCl_2 . The concentration of membrane protein was 1.7 mg/mL , and titrations were carried out up to a total NPG concentration of 0.5 mM for DTT-treated vesicles and 1 mM for plumbagin-treated vesicles in the absence or presence of HgCl_2 , respectively. The curves are calculated with eq A15 (Appendix). The parameter values are listed in Table I (preparation 2).

over RSO membrane vesicles and were not further investigated.

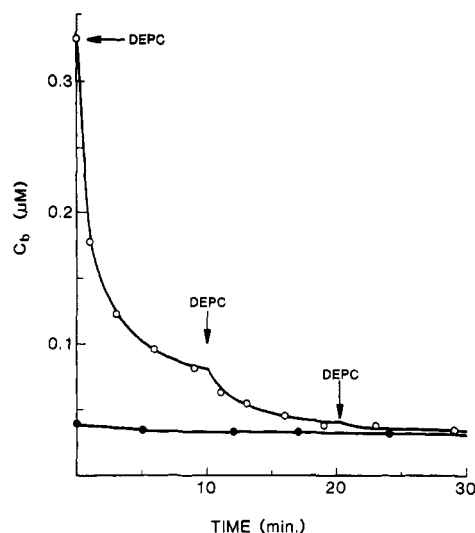


FIGURE 6: Effect of DEPC treatment on NPG binding to purified RSO membrane vesicles at pH 7.5. At times 0, 10, and 20 min, DEPC (final concentration 3 mM) was added to a suspension of vesicles (5.2 mg/mL) containing 2 μ M NPG. At the indicated times, 90- μ L samples were transferred to Eppendorf tubes and rapidly mixed with 9 μ L of a 100 mM histidine solution in order to inactivate DEPC (Melchior & Fahrney, 1970). The samples received 1 μ L of water (○) or 1 μ L of a 25 mM HgCl_2 solution (●).

NPG Binding to Chemically Modified *lac* Permease. Treatment of *lac* permease with NEM or plumbagin did not completely abolish specific binding of NPG (Figure 3). RSO membrane vesicles incubated with 1, 3, or 10 mM NEM for 1 h displayed the same binding of NPG. Similarly, incubation with 10, 30, or 100 μ M plumbagin yielded the same level of reduced binding (data not shown). This rules out the possibility that the residual binding arises from a fraction of unmodified enzyme molecules. Titrations of NPG binding to permease alkylated with NEM [Figure 4 (●)] or oxidized with plumbagin [Figure 5 (●)] demonstrated that the modifications reduced the binding affinity. Analysis of the binding data up to total concentrations of 500 μ M in terms of only one site resulted in a K_L of about 190 μ M both for the alkylated and for the oxidized enzyme. The chemical modification had little or no effect on the binding capacity (Table I). Parameter estimation was then carried out simultaneously with the binding data for untreated and chemically modified membranes, assuming the same binding capacity for both types of membranes. In the case of plumbagin-treated vesicles, this procedure yielded a dissociation constant for the low-affinity site of about 8 mM, albeit with a rather large standard deviation (Table I, preparation 2). Therefore, this value is only an approximation. The same procedure applied to the data for the NEM-treated vesicles did not work.

Figure 6 demonstrates that all specific binding of NPG to RSO membranes is abolished upon treatment with the histidine reagent DEPC. The slowing down of the initially fast decrease in binding was mainly due to hydrolysis of DEPC; adding new DEPC immediately speeded up the process again. Adding enough DEPC to give all enzyme molecules a chance to react with the reagent before it was totally hydrolyzed eventually led to the same binding as found in the HgCl_2 -treated membranes.

***lac* Permease Has Two Binding Sites for NPG.** Proteoliposomes offer two advantages over membrane vesicles: (i) choosing a high protein to lipid ratio in the reconstitution step results in a high permease concentration in the pellet and a low nonspecific binding; (ii) the concentration of *lac* permease can be determined by means of quantitative amino acid

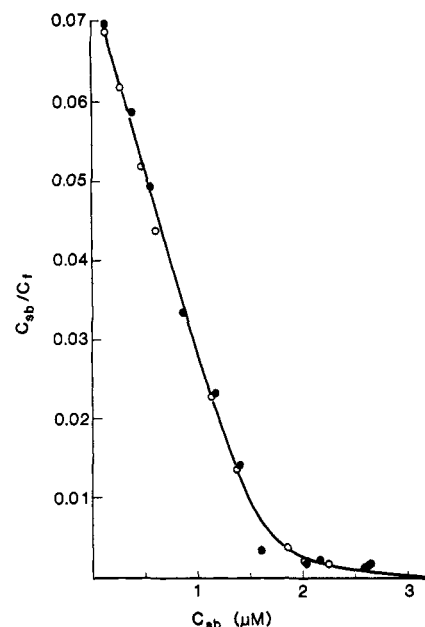


FIGURE 7: Scatchard plot of NPG binding to purified *lac* permease reconstituted into proteoliposomes at pH 7.5. The values for the concentration of NPG bound to *lac* permease and for the free ligand were calculated from two independent titrations (○ and ●) up to 2 mM total NPG concentration with the same proteoliposome preparation. For the assessment of nonspecific binding, see Materials and Methods. The concentration of *lac* permease was $1.63 \pm 0.05 \mu\text{M}$ as determined by quantitative amino acid analysis. The curve was calculated with eq A15 (see Appendix) and the parameter values listed in Table I (preparation 5).

analysis. The data obtained for binding of NPG to untreated proteoliposomes were similar to the binding to RSO membrane vesicles. However, the considerably lower nonspecific binding together with the high content of permease in the membranes enabled us to collect binding data with reasonable accuracy up to 2 mM total NPG concentration. Nonspecific binding was measured both to HgCl_2 - and to DEPC-treated proteoliposomes over the same concentration range (see Materials and Methods). Figure 7 presents a Scatchard plot of two sets of binding data (○ and ●) which were obtained with the same proteoliposome preparation but in two independent experiments. This demonstrates the high reproducibility of the data. The deviation from a straight line for bound concentrations larger than 1.5 μ M is very clear-cut and proves the existence of a second binding site. Estimation of the parameters yielded dissociation constants of 21.2 μ M and 1.6 mM (Table I, preparation 5). The binding capacity per site amounts to $1.56 \pm 0.03 \mu\text{M}$ which, together with the permease concentration of $1.63 \pm 0.05 \mu\text{M}$ determined by quantitative amino acid analysis, results in a stoichiometry of 1.9 ± 0.1 molecules of NPG bound per molecule of permease. Therefore, *lac* permease has two binding sites for NPG. Table I lists values of binding parameters for a second proteoliposome preparation (6). In this case, the parameter values for nonspecific binding in the presence of HgCl_2 were quite different from those for preparation 5. It should then be kept in mind that the parameters for the nonspecific binding are primarily operational parameters which cannot necessarily be interpreted in terms of capacity and affinity for nonspecific binding. The parameter values for specific binding, however, were in good agreement.

DISCUSSION

The improvement achieved with the protocol presented here for measuring binding of ligands to *lac* permease as compared to flow dialysis (Rudnick et al., 1976) or analyzing the pellet

only (Kennedy et al., 1974) is best illustrated by the titrations of proteoliposomes. Using as little as 1–2 μM permease, we could estimate binding for total NPG concentrations up to 2 mM. In contrast, inferring the bound ligand from the depletion in the supernatant, which is equivalent to flow dialysis, failed for total concentrations above 10 μM , and about 5–10-fold larger concentrations of permease would be required to allow for a reliable assessment of only the high-affinity binding of NPG.

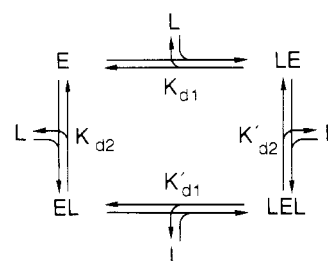
Chemical modifications of *lac* permease cause different effects on its binding properties. Alkylation with NEM or oxidation with plumbagin (the target is a cysteine residue at position 148', Beyreuther et al., 1981) abolishes the catalytic activity of the permease (Fox et al., 1965; Cohn et al., 1981). We have demonstrated that this coincides with about a 10-fold decrease in affinity of the permease for NPG with no effect on the binding capacity. This is at variance with the findings of Neuhaus and Wright (1983), who reported no change in the high-affinity site but a loss in binding sites. The lower sensitivity and accuracy of the binding assay used by these authors may have led to this misconception; although the method was not specified, it was most likely flow dialysis. We could not unambiguously define the effect of alkylation and oxidation of the enzyme on the second site.

The loss of activity upon treatment of *lac* permease with diethyl pyrocarbonate (Padan et al., 1979) may be caused by a loss of affinity of the enzyme for its substrates. We could not detect any binding of NPG to DEPC-treated proteoliposomes above the binding in the presence of HgCl_2 . It was reported that DEPC treatment of *lac* permease in *E. coli* strains ML308-225 (Padan et al., 1979), T206, or T217 (Neuhaus and Wright, 1983) has little or no effect on the high-affinity NPG binding site but decreases the number of sites. Thus, e.g., a 10-min incubation with 3 mM DEPC at pH 6 caused a 25% loss for energized ML308-225 RSO vesicles while nonenergized T206 vesicles and T217 cells lost all and 70% of their binding sites, respectively. Both investigations did not take into account that DEPC is readily hydrolyzed (Berger, 1975) as demonstrated in Figure 6. In fact, hydrolysis occurs at rates comparable to those for the reaction of DEPC with *lac* permease (Püttner, Walz, and Kaback, unpublished results). Hence, a different reactivity of the enzyme in different strains and under different experimental conditions could explain the differences in loss of binding sites. This is in line with the finding of Neuhaus and Wright (1983) that T217 cells incubated with 8 mM DEPC for 10 min lost 90% of the binding sites.

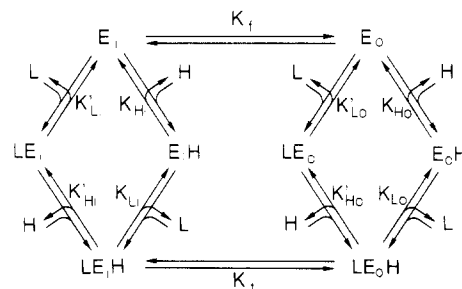
Our data strongly support the notion that *lac* permease has two binding sites for NPG. The first site has a dissociation constant of 16 μM at pH 7.5 and room temperature in cytoplasmic membrane vesicles, in fair agreement with values reported before (Kennedy et al., 1974; Rudnick et al., 1976; Wright et al., 1981). The somewhat higher values in proteoliposomes are probably caused by the high permease content of these membranes. The second binding site has a dissociation constant of about 1.6 mM at pH 7.5 and was detected here for the first time. The affinity of this site may seem very low relative to the first site, but it should then be kept in mind that the affinity for the natural substrate lactose is in the millimolar range as well. The two binding sites should not be mistaken for the two sites (class I and class II) postulated by Fox and Kennedy (1965) which imply that a given ligand can bind only to one of the two sites.

The binding of two ligand molecules (L) to an enzyme (E) in general terms involves the steps shown in Scheme I. Here

Scheme I



Scheme II



LE, EL, and LEL are the species arising when L is bound to the first, to the second, and to both sites, respectively. K_{d1} and K_{d2} denote the dissociation constants for binding of L to the first and the second site of the free enzyme, respectively, while the corresponding primed constants pertain to binding to the enzyme with the other site occupied. Thermokinetic balancing (Walz & Caplan, 1988) requires that $K_{d1}K'_{d2} = K'_{d1}K_{d2}$. It can be derived that the phenomenological dissociation constants K_{L1} and K_{L2} as determined in a ligand titration experiment are related to the intrinsic dissociation constants as follows:

$$K_{L1/2} = (K'_{d1} + K'_{d2})/2 \pm \{(K'_{d1} + K'_{d2})^2/4 - K_{d1}K'_{d2}\}^{1/2}$$

In general, it is impossible to resolve the experimental K_{L1} 's into the intrinsic dissociation constants in Scheme I except for the special case of no interaction between the two sites; then $K_{L1} = K_{d1}$ and $K_{L2} = K_{d2}$. However, the implications for the performance of *lac* permease resulting from the finding that one permease molecule has two substrate binding sites can be discussed qualitatively.

Both sites could be attributed to the transport cycle of the enzyme. This would mean that the enzyme can transport two ligands per proton. Stoichiometry measurements rule out this possibility (Patel et al., 1982; Page et al., 1988). Then, the two binding sites could reflect two independent catalytic cycles or one cycle whose rate constants are modulated by binding of ligand to a regulatory site. The cycles should transport one ligand per proton as is the case for Scheme II which was proposed by Page (1987). The cycle proposed by Kaback (1986) is a special case of this scheme; the ligand binds only to the protonated carrier ($K'_{Lo} \gg K_{Lo}$, which implies $K'_{Ho} \ll K_{Ho}$ due to thermokinetic balancing). When analyzing Scheme II under equilibrium conditions, i.e., no membrane potential exists, and external and internal concentrations are equal for ligand and proton, it can be shown that binding of L is governed by *only one* phenomenological dissociation constant. It comprises all the intrinsic constants in the scheme and in general depends on pH. Data recently obtained in this laboratory on proton binding to *lac* permease clearly indicate that the phenomenological dissociation constant should depend on pH in the range from 5.5 to 8.5 (Püttner, Walz, and Kaback, unpublished results), but a similar dependence of the high-affinity NPG binding site on pH was not found [see also

Wright et al. (1981)]. This leads to the conclusion that the binding of NPG to the first site cannot be associated with a catalytic cycle of *lac* permease. Unfortunately, our binding assay is not accurate enough to establish a clear-cut pH dependence for the second site.

Two kinetic regimes with different values for K_m and V_{max} are evident in Eadie-Hofstee plots of the initial rates of lactose uptake in the presence of an electrochemical potential difference for protons. The regime with the higher K_m seems to be only weakly affected by the protonmotive force; the other shows a strong dependence of V_{max} with little effect on K_m (Robertson et al., 1980). The explanation proposed for this phenomenon, i.e., a protonmotive force induced dimerization of the permease, has recently been disproved (Costello et al., 1987). Page (1987) has shown that the cycle depicted in Scheme II can mimic the observed phenomena under certain conditions, however, this explanation becomes unlikely in view of the following data (Lolkema et al., 1990). The rate of exchange of labeled lactose was measured at pH 5.5 and in the absence of a protonmotive force, i.e., conditions where the turnover of the catalytic cycle is essentially blocked. Eadie-Hofstee plots of the exchange rate at constant internal but variable external concentration of lactose display a two-regime behavior similar to that of active transport even in the case of the mutant Glu325-Ala which cannot perform active transport at all (Carrasco et al., 1986). Similar experiments with a constant external but variable internal lactose concentration show only one kinetic regime. We postulate that the underlying mechanism of the two kinetic regimes in both active transport and the exchange reaction is the same and involves binding of ligand to the two sites demonstrated in Figure 7. The shift between the two regimes is caused by binding of lactose to a regulatory site which is accessible only from the external medium. The binding site with $K_{L1} = 16 \mu\text{M}$ for NPG would then be the regulatory site because of its insensitivity to pH. The binding site with $K_{L2} = 1.6 \text{ mM}$ for NPG would reflect the binding to the catalytic cycles.

The physiological significance of a regulated lactose uptake may relate to an economic expenditure of metabolic energy. Carrier-catalyzed diffusion may be sufficient when a cell faces a high enough lactose concentration. Hence, the catalytic cycle which is active in case of an occupied regulatory site need not be coupled to proton movement. Uptake driven by the electrochemical potential difference for protons, which has to be generated by the cell, may be required when the lactose concentration in the surroundings becomes too small for a high enough rate of lactose influx. This mechanism comes into play because a decreasing lactose concentration lowers the occupancy of the regulatory site which also gradually switches on the coupling of the proton flux to the lactose flux.

ACKNOWLEDGMENTS

This investigation was carried out in the laboratory of Dr. H. Ronald Kaback. We thank him for his hospitality, continuous support, and many stimulating discussions.

APPENDIX

Analysis of the Binding Assay. The total concentration of ligand, c_t , in the membrane suspension of volume V_t is the sum of the concentrations of bound and free ligand, c_b and c_f , respectively:

$$c_t = c_b + c_f \quad (\text{A1})$$

Spinning down the vesicles leaves only the free ligand in the supernatant. The pellet contains all the bound ligand with mole number $c_b V_t$, and a fraction of the initially present free

ligand with mole number $c_f V_p$ as determined by the pellet volume V_p . Removing the supernatant and resuspending the pellet in a volume V_{pd} of fresh buffer without ligand create the concentration c_{pr} in the volume $V_{pd} + V_p$ of the pellet resuspension, where

$$c_{pr} = (c_b V_t + c_f V_p) / (V_{pd} + V_p) \quad (\text{A2})$$

Elimination of c_f from eq A1 and A2 yields

$$c_b = \{c_{pr}(V_{pd}/V_p + 1) - c_t\} / (V_t/V_p - 1) \quad (\text{A3})$$

The volume of the pellet is estimated by means of a marker substance M which is added at a concentration c_M to the total sample. Its concentration in the supernatant and the pellet is also c_M while in the pellet resuspension

$$c_{Mpr} = c_M V_p / (V_{pd} + V_p) \quad (\text{A4})$$

The concentrations c_{pr} and c_{Mpr} are experimentally determined by means of double labeling of ligand and marker. Then, in terms of the dpm values d_{Yx} for substance Y (ligand L or marker M) found in the volume V_{xc} taken for counting (cf. Figure 1) from the total sample ($x = t$), the supernatant ($x = s$), and the pellet resuspension ($x = p$), respectively:

$$c_{pr} = c_t V_{tc} d_{Lp} / V_{pc} d_{Lt} = (c_b - c_t) V_{sc} d_{Lp} / V_{pc} d_{Ls} \quad (\text{A5})$$

and

$$c_{Mpr} = c_M V_{xc} d_{Mp} / V_{pc} d_{Mx} \quad x = t \text{ or } s \quad (\text{A6})$$

Solving eq A4 for V_p yields by virtue of eq A6

$$V_p = V_{pd} / (d_{Mx} V_{pc} / d_{Mp} V_{xc} - 1) \quad x = t \text{ or } s \quad (\text{A7})$$

Insertion of eq A5 and A7 into eq A3 yields

$$c_b = c_t(r_t - 1) / (F_t - 1) = c_t(r_s - 1) / (F_s + r_s - 1) \quad (\text{A8})$$

with the abbreviations

$$r_x = d_{Lp} d_{Mx} / d_{Lx} d_{Mp}$$

and

$$F_x = (V_t/V_{pd})(d_{Mx} V_{pc} / d_{Mp} V_{xc} - 1) \quad x = t \text{ or } s$$

Finally, from eq A1 and in terms of the dpm values found for the ligand in the total sample and the supernatant:

$$c_b = c_t(1 - d_{Ls} V_{tc} / d_{Lt} V_{sc}) \quad (\text{A9})$$

which estimates the concentration of bound ligand from the depletion in the supernatant.

Evaluation of Nonspecific Binding. Binding of NPG to lipids and proteins in the membrane is determined by a multitude of dissociation constants with pertinent capacities. For the present purpose, it is sufficient to approximate the concentration of nonspecifically bound NPG, c_{ub} , by means of an "average" dissociation constant K_{ub} and an "average" capacity C_{ub} :

$$c_{ub} = k_{ub} c_f / (1 + c_f / K_{ub}) \text{ where } k_{ub} = C_{ub} / K_{ub} \quad (\text{A10})$$

If the total concentration of NPG is small compared to K_{ub} , then $c_f \ll K_{ub}$, and eq A10 reduces to

$$c_{ub} \approx k_{ub} c_f = k_{ub} c_t / (1 + k_{ub}) \text{ for } c_t \ll K_{ub} \quad (\text{A11})$$

Note that the right-hand side of eq A11 applies only to cases without specific binding. The conditions $c_t \ll K_{ub}$ was found to hold for all membrane preparations up to $c_t = 500 \mu\text{M}$. If titrations were extended beyond this value, a slight curvature in the dependence of c_{ub} on c_t was observed, and eq A10 instead of eq A11 had to be used.

Evaluation of Binding to Two Sites. In case of two binding sites with equal capacity C_{sb} , it follows for the concentration

of specifically bound ligand c_{sb} :

$$c_{sb} = C_{sb} \{ 1 / (1 + K_{L1}/c_f) + 1 / (1 + K_{L2}/c_f) \} \quad (A12)$$

K_{L1} and K_{L2} are the phenomenological dissociation constants for the two sites. Substituting c_{sb} (eq A12) and c_{ub} (eq A11) into

$$c_b = c_{sb} + c_{ub} \quad (A13)$$

and introducing the resulting equation into eq A1 yield upon rearranging a cubic equation for c_f whose only root satisfying the condition $0 < c_f < c_t$ is

$$c_f = 2a^{1/2} \cos \{ \cos^{-1} (b/a^{3/2}) / 3 \} - p \quad (A14)$$

with

$$a = p^2 - q/3$$

$$b = p(q/2 - p^2) + c_t K_{L1} K_{L2} / [2(1 + k_{ub})]$$

where

$$p = [(2C_{sb} - c_t) / (1 + k_{ub}) + K_{L1} + K_{L2}] / 3$$

$$q = (C_{sb} - c_t)(K_{L1} + K_{L2}) / (1 + k_{ub}) + K_{L1} K_{L2}$$

Combining eq A11–A14 yields c_b as a function of c_t which was used to estimate values for the parameters C_{sb} , K_{L1} , and K_{L2} by means of the nonlinear fitting program as described under Materials and Methods.

Rearranging eq A12 yields a quadratic equation for c_f with only one positive root from which the following dependence of c_{sb}/c_f on c_{sb} is obtained:

$$c_{sb}/c_f = (2C_{sb} - c_{sb})c_{sb} / \{ h + [h^2 + (2C_{sb} - c_{sb})c_{sb}K_{L1}K_{L2}]^{1/2} \} \quad (A15)$$

with the abbreviation

$$h = (K_{L1} + K_{L2})(c_{sb} - C_{sb})/2$$

Using eq A15 in the nonlinear fitting program and the data of the Scatchard plots gives an alternative way to estimate parameter values.

Registry No. NPG, 7493-95-0; L-Cys, 52-90-4; lactose permease, 9068-45-5.

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